Title: Enrichment of maternal diet with conjugated linoleic acids influences desaturases activity and fatty acids profile in livers and hepatic microsomes of the offspring with 7,12-dimethylbenz[a]antracene-induces mammary tumors

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ENRICHMENT OF MATERNAL DIET WITH CONJUGATED LINOLEIC ACIDS INFLUENCES DESATURASES ACTIVITY AND FATTY ACIDS PROFILE IN LIVERS AND HEPATIC MICROSOMES OF THE OFFSPRING WITH 7,12-DIMETHYLBENZ[A]ANTHRACENE-INDUCED MAMMARY TUMORS

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Abstract: The aim of this study was to assess the influence of diet supplementation of pregnant and breast-feeding female Sprague-Dawley rats with conjugated linoleic acids (CLA) on the Δ6- and Δ5-desaturase activity in hepatic microsomes as well as on fatty acids profile and lipids peroxidation in liver and hepatic microsomes of the progeny with chemically induced mammary tumors. Rats were divided into two groups with different diet supplementation (vegetable oil (which did not contain CLA) or CLA). Their female offspring was divided within these groups into two subgroups: (1) – fed the same diet as mothers (K1 – oil, O1 – CLA), and (2) – fed the standard fodder (K2, O2). At 50th day of life, the progeny obtained carcinogenic agent (7,12-dimethylbenz[a]anthracene). Higher supply of CLA in diet of mothers resulted in lower susceptibility to chemically induced mammary tumors in their offspring ($p = 0.0322$). It also influenced the fatty acids profile in livers and in hepatic microsomes, especially polyunsaturated n3 and n6 fatty acids. CLA inhibited the activity of the desaturases, which confirmed that CLA can reduce the level of arachidonic acid directly, reducing linoleic acid content in membranes, or indirectly, through the regulation of its metabolism. We were unable to confirm or deny the antioxidative properties of CLA. Our results indicate that the higher supply of CLA in mothers' diet during pregnancy and breastfeeding causes their incorporation into tissues of children, changes the efficiency of fatty acids metabolism and exerts health-promoting effect in their adult life reducing the breast cancer risk.

Keywords: conjugated linoleic acids, Δ5-desaturase, Δ6-desaturase, breast cancer, rats

Abbreviations: AA – arachidonic acid, ALA – α-linoleic acid, CLA – conjugated linoleic acids, D5D – Δ5-desaturase, D6D – Δ6-desaturase, DHA – docosahexaenoic acid, DMBA – 7,12-dimethylbenz[a]anthracene, EPA – eicosapentaenoic acid, FA – fatty acids, FAME – fatty acids methyl esters, LA – linoleic acid, MDA – malonyldialdehyde, OL – oleic acid, RA – rumenic acid, TBARS – thiobarbituric acid reactive substances, VA – vaccenic acid

Dietary fat is the most concentrated source of energy for organism as 1 g of fat provides 9 kcal of energy. It is also the carrier of fat-soluble vitamins and essential fatty acids. Liver plays an important role in the metabolism of lipids. It produces the bile, which facilitates the digestion and intestinal absorption of lipids. The synthesis and oxidation of fatty acids take place in liver. Formation of ketone bodies from fatty acids also occurs in liver. Moreover this organ integrates the synthesis and metabolism of plasma lipoproteins. Desaturases are the enzymes which are involved in transformation of fatty acids. They can insert the double bonds into different positions of fatty acids (Δ4, Δ5, Δ6, Δ9) but not higher than Δ9, and that is why linoleic (LA) and α-linolenic (ALA) essential fatty acids should be supplied with diet (1).

Breast cancer is the most frequent type of cancer among women and the third in global population. Numerous nutritional factors are associated with elevated or reduced risk of this type of cancer (2, 3). Despite the fact that the etiology of most cases of this disease is unknown (4), quantity and quality of fat, especially the fatty acids ratio in diet,

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are associated with many cancers (e.g., breast, colon) (3).

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid which contain two conjugated double bonds in their chain. They occur especially in milk, dairy products and meat from ruminants. These types of food are the richest dietary sources of CLA. *Cis*-9, *trans*-11 octadecadienoic acid (rumenic acid – RA) constitutes over 90% of all CLA isomers in dietary products (5). The second important CLA isomer – *trans*-10, *cis*-12 octadecadienoic acid, is present in many dietary supplements (6). Despite the fact, that rumenic acid can be endogenously synthesized from *trans*-11 octadecenoic acid (vaccenic acid – VA) by the action of Δ9-desaturase, dietary intake is the main source of CLA for people (7). Results of scientific research show the positive impact of CLA in different pathological conditions, especially different types of cancer, e.g., breast cancer (7–9). CLA are active in each step of cancer development, from initiation to metastasis. They can reduce the cancerous process risk and there are many possible mechanism of this action: antioxidative properties, influence on eicosanoids synthesis, apoptosis etc. (11).
Polyunsaturated fatty acids have beneficial effect in many pathological conditions, e.g., in many types of cancer. The content of unsaturated fatty acids in cellular structures is the result of intake of essential polyunsaturated fatty acids from diet and their endogenous synthesis and subsequent utilization in body building. Elongation and desaturation depend on both the absolute content of these fatty acids and the activity of various enzymes, e.g., desaturases, which control this metabolic pathway. Activity of Δ6- and Δ5-desaturases is the main factor controlling the conversion of dietary linoleic acid (LA) to arachidonic acid (AA). Therefore, in the examination of fatty acids composition in mammalian tissues it is essential to take into account not only diet but also the activity of crucial enzymes. Many dietary factors can influence this activity (12–14).

Table 1. Fatty acids composition of applied diets (% of FA).

Fatty acid	Fodder	Fodder + oil	Fodder + CLA
C6:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C12:0	< 0.1	< 0.1	0.1 ± 0.1
C14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C15:0	0.1 ± 0.0	0.1 ± 0.0	–
C16:0	13.3 ± 0.1	10.5 ± 0.1	11.2 ± 0.2
C16:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C17:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C18:0	2.7 ± 0.0	2.5 ± 0.0	2.5 ± 0.0
C18:1 n9 *cis*	16.6 ± 0.1	34.5 ± 0.1	15.9 ± 0.2
C18:2 n6 *cis*	40.5 ± 0.1	35.1 ± 0.1	31.5 ± 0.3
C18:3 n3	22.7 ± 0.1	14.1 ± 0.0	16.7 ± 0.2
C20:0	0.2 ± 0.0	0.2 ± 0.1	–
cis-9, *trans*-11 CLA	–	–	8.6 ± 0.3
trans-10, *cis*-12 CLA	–	–	8.5 ± 0.4
C20:1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:2	–	–	0.1 ± 0.0
C22:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
C20:5 n3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C22:2	0.2 ± 0.0	0.1 ± 0.0	–
C23:0	–	–	0.1 ± 0.0
C24:0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
C24:1	< 0.1	0.1 ± 0.0	–

The aim of this study was to assess the influence of diet supplementation of pregnant and breastfeeding female Sprague-Dawley rats with conjugated linoleic acids on thiobarbituric acid reactive substances (TBARS) concentration and fatty acids profile in the liver and hepatic microsomes as well as on the Δ6- and Δ5-desaturase (E.C. 1.14.19.) activity in hepatic microsomes of the progeny with chemically induced mammary tumors.

EXPERIMENTAL

Animals

This research and guiding principles in the care and use of laboratory animals were approved by The Local Ethical Committee on Animal Experiments. The whole experiment was thoroughly described previously (15). Briefly, maiden adult female Sprague-Dawley rats (n = 8), which came from Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Warszawa, Poland), after 1-week adaptation period were randomly divided into two groups of 4 each. Each group had a different dietary supplementation: group K received vegetable oil whereas group O – Bio-CLA (Pharma Nord, Vojens, Denmark) given intragastrically in the amount 0.15 mL per day, which corresponds to 100 mg of CLA. Applied vegetable oil, purchased from the Pharma Nord, did not contain the CLA and was a substrate for the CLA synthesis. Detailed composition of fatty acids of applied oil was: C16:0 (5.7 ± 0.0)%, C18:0 (0.4 ± 0.0)%, C18:1 n9 (64.5 ± 0.1)%, C18:2 n6 (25.5 ± 0.0)%, C20:04 n6 (0.3 ± 0.0)% C24:1 (0.2 ± 0.0)%. Supplementation lasted for the whole period of pregnancy and breastfeeding. The female offspring was separated from their mothers at the 30th day of age and within each group of supplementation was divided into two subgroups of 8–10 individuals each: 1 – with diet enriched with the same dietary supplement that had been previously given to their mothers (group K1 obtained vegetable oil and group O1 – Bio-C.L.A. and 2 – fed exclusively the standard Labofeed H fodder H (fodder producer "Morawski", Żurawia 19, Kcynia, Poland). Table 1 shows the fatty acids composition of applied diets. Dietary supplementation of subgroups was conducted for the following 21 weeks from 30 days of life. The offspring received *via* gavage at 50th day of life a single dose of 80.00 mg/kg body weight of carcinogenic agent – DMBA (7,12-dimethylbenz[a]anthracene, approx. 95%, Sigma-Aldrich, Saint Louis, Missouri, USA) for the induction of mammary tumors. Rats were weighed and palpated weekly to detect the appearance of mammary tumors. The animals were decapitated and exsanguinated, and livers and tumors were collected. Isolated tumors were histopathologically evaluated as adenocarcinomas and papillary adenocarcinomas of mammary gland. Spontaneous tumors were not found in maternal rats during the whole experiment.

Preparation of experimental material

Samples of liver were collected during necropsy and stored at –70°C before the analysis.

Hepatic microsomes were prepared immediately after decapitation according to the slightly modified method of Kłyszejko-Stefanowicz (16). A sample of liver (4 g) was mechanically homogenized in saccharose solution (16 mL; 0.25 mol/L) buffered with Tris-buffer (pH 7.4). The obtained homogenate was centrifuged three times: (1) for 10 min at 1000 × g at 4°C, (2) for 20 min at 16000 × g at 4°C, and (3) for 75 min at 100000 × g at 4°C. The applied modification refers to the fact, that as we were interested only in microsomal fraction, each time the sediment was discarded and the supernatant was again centrifuged with increasing speed. Final pellet was resuspended in 4 mL 0.25 mol/L saccharose solution and obtained suspension of hepatic microsomes was stored at –70°C until further analysis.

Fatty acids analysis

Fatty acid analysis was made with gas chromatography (GC) using gas chromatograph (GC-17A gas chromatograph, Shimadzu, Kyoto, Japan) with capillary column (BPX 70; 60 m × 0.25 mm i.d., film thickness 0.20 μm, SGE, Ringwood, Australia) and flame-ionization detection.

Samples of liver were thawed only once and three parallel samples of 0.2 g were taken for lipids extraction according to Folch et al. with slight modification (17). Applied modification refers to the volume and sequence of organic solvents, which were used in extraction procedure. Fatty acids methyl esters (FAME) were prepared according to procedure of Bondia-Pons et al. (18) with slight modifications, which were thoroughly described elsewhere (19).

The hepatic microsomes were thawed only once and three parallel samples of 200 μL of microsomal suspensions were taken for lipids extraction. Lipids were extracted according to Folch et al. with slight modification (17). Briefly, a sample of microsomal suspension was mixed with 2.5 mL of chloroform : methanol (2 : 1, v/v) and after vigorous shaking the chloroform layer was separated. The residue

was mixed with 1.5 mL of chloroform : methanol (2 : 1, v/v) and the extraction was repeated. Combined chloroform layers were centrifuged for 10 min at 1000 × g and the sediment was discarded. The organic extract was evaporated under stream of nitrogen and the residue was taken for the preparation of FAME according to procedure of Bondia-Pons et al. (18) with slight modifications, which was also used for liver samples. The detailed procedure of FAME analysis was previously described and applied modification refers to the volumes of solvents (19).

TBARS analysis

Samples of liver were thawed only once and sample of 0.5 g was taken for TBARS analysis with spectrophotometric method (20). This sample was mechanically homogenized with teflon homogenizer in 2.5 mL of sodium chloride solution (0.9%). Afterwards, 2.5 mL of phosphate buffer (pH 7.0) and 1.5 mL of trichloroacetic acid (1.7 mol/L), were added to 0.5 mL of previously obtained homogenate. The whole sample was shaken vigorously and centrifuged for 15 min at 1000 × g at 0°C, to separate the protein sediment. The 1.0 mL of 2-thiobarbituric acid solution (69 mmol/L) was added to 3.0 mL of supernatant; the whole sample was shaken vigorously and heated for 15 min on boiling water bath. The absorbance was measured at $\lambda = 530$ nm after cooling to room temperature. The reference sample was prepared analogously with 0.5 mL of sodium chloride solution (0.9%). Three parallel samples were prepared for each liver sample.

Enzymes activity analysis

Enzymes activity was determined in an indirect way because the amount of AA formed *in vitro* from LA correlates with the activity of the investigated enzymes (21). The measurement of these activities was carried out according to previously published method (22), with slight modifications. We decided not to use the radioisotopes, which were applied in original procedure. Each 1.0 mL reaction mixture contained 5 mmol ATP, 0.1 mmol CoA, 1.25 mmol NADH, 0.5 mmol niacinamide, 2.25 mmol glutathione and 5 mmol $MgCl_2$, dissolved in phosphate buffer pH 7.4 and 200 nmol sodium salt linoleic acid. The reaction mixture was preincubated for 5 min at 37°C. The enzymatic reaction was started by the addition of 0.2 mL of hepatic microsomes suspension. The whole mixture was incubated in a shaking water bath for 90 min at 37°C. Further analytical procedure consisted of lipids extraction according to Folch et al. (17) and esterification (22).

The fatty acids concentrations in samples were determined with high performance liquid chromatography (HPLC) with UV/VIS detection (Merck Hitachi, L-7100 pump, UV/VIS L-74200 detector, CTO-10 AS oven, YMC-Pack ODS-AM S-5 μm column, the column temp. 30°C, $\lambda = 198$ nm). The activity index of Δ6-desaturase (D6D) was calculated as the ratio of γ-linolenic acid (GLA) concentration to linoleic acid (LA) concentration and the activity index of Δ5-desaturase (D5D) was expressed as the ratio of arachidonic acid (AA) concentration to dihomo-γ-linolenic acid (DGLA) concentration in liver microsomes. The content of AA in liver microsomes was calculated in protein content, determined using the method of Lowry et al. (23). The differences in AA concentrations between incubated and non-incubated samples indicate the activity of investigated enzymes.

Table 2. Characterization of experimental groups.

Group (number of individuals)	K1 (n = 10)	K2 (n = 9)	O1 (n = 8)	O2 (n = 9)
Mothers' diet	Lab. H + oil	Lab. H + oil	Lab. H + CLA	Lab. H + CLA
Progeny's diet	Lab. H + oil	Lab. H	Lab. H + CLA	Lab. H
Number of individuals with tumors	8	7	2	3
Age of first tumor appearance [week]	19	17	15	21
Total number of tumors	14	11	4	5
Maximal number of tumors per individual	0–3	0–4	0–3	0–2
Maximal weight of tumors per individual [g]	31.77	16.19	11.81	4.27

Table 3. Comparison of TBARS concentration in livers of investigated groups.

	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
Liver [g]	7.21 ± 1.31	5.98 ± 0.59	6.65 ± 0.58	6.15 ± 0.58	0.0079
Liver *vs.* total body weight [%]	3.2 ± 0.7	2.7 ± 0.2	3.0 ± 0.2	2.8 ± 0.3	0.0224
Fat [%]	2.6 ± 0.4	2.8 ± 0.4	2.3 ± 0.4	2.3 ± 1.0	0.0094
TBARS [nM/g of tissue]	60.10 ± 30.86	46.04 ± 44.25	53.95 ± 47.36	50.03 ± 37.00	NS
TBARS [μM/g of fat]	2.39 ± 1.39	1.63 ± 1.55	2.46 ± 2.18	2.84 ± 2.31	NS

All data are shown as the mean values ± standard deviation; p value < 0.05 for variables with significant differences among groups in Kruskal-Wallis test; NS – not significant differences among groups in Kruskal-Wallis test (p value > 0.05).

Statistical analysis

All data are shown as the mean values ± standard deviation. For variables with skew distribution, obtained data were transformed into logarithms and retransformed after calculations. They are presented as the mean and confidence interval. Results obtained for fatty acids and TBARS content were evaluated with Statistica 10.0 (StatSoft, Kraków, Poland). Due to the relatively small number of individuals in each group, the data were tested with Kruskal-Wallis test and p-value ≤ 0.05 was considered significant.

Results of enzymes activity were evaluated with statistical methods such as: PCA (principal component analysis), ANOVA with permutation test and ASCA (analysis of variance-simultaneous component analysis).

RESULTS

All four groups of the progeny received DMBA administered intragastrically in a single dose of 80 mg/kg body weight and the breast tumors, identified as adenocarcinomas and papillary adenocarcinomas of mammary gland, appeared in all investigated groups. The characterization of experimental groups, as well as the effectiveness of cancer induction are shown in Table 2. CLA decreased the incidence of mammary cancer in investigated animals (p = 0.0322). Moreover, the tumors' number and weight were smaller than in two oil groups. In O1 breast tumors appeared much earlier (on average at 15th week of life) than in other groups, but their number per individual was the smallest. We observed strong cancer preventive properties of CLA also in O2 group, where both the number and mass of tumors were similar to those in O1, which suggests that dietary intake of CLA from the very early period of life is very important for reducing the risk of carcinogenesis (15).

Comparison of average mass of livers revealed that applied supplementation significantly influenced their weight (Table 3). We observed the highest mass of livers obtained from animals from K1, whereas the mass of these organs from K2 was the smallest. Two-step supplementation with CLA seems not to affect the weight of examined organs, as their mass was slightly lower than in K1 but slightly higher in O2 and K2.

We compared the fat content in livers from examined groups. There were significant differences in amount of fat among them (Table 3). The highest content of fat occurred in livers from K2, whereas its content in both CLA groups was lower. Mean con-

Table 4. Fatty acids profile in livers of experimental groups.

Fatty acid	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
	[% of FA]				
C12:0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	NS
C14:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	NS
C15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.002
C16:0	18.1 ± 1.5	19.1 ± 1.7	16.8 ± 0.5	19.5 ± 1.1	0.001
C16:1	0.4 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.000
C17:0	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	NS
C17:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:0	23.0 ± 2.1	21.4 ± 2.0	25.2 ± 1.0	21.1 ± 1.3	0.000
C18:1 n9 *cis*	5.8 ± 1.2	5.5 ± 1.5	4.4 ± 0.7	5.6 ± 1.1	0.042
C18:2 n6 *trans*	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.000
C18:2 n6 *cis*	15.6 ± 2.3	16.1 ± 1.5	13.4 ± 1.3	17.0 ± 1.6	0.003
C18:3 n6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	NS
C18:3 n3	0.7 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	0.000
cis-9, *trans*-11 CLA	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.000
trans-10, *cis*-12 CLA	nd	nd	0.1 ± 0.0	nd	NS
C20:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C21:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.015
C20:2	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.016
C20:4 n6	18.3 ± 2.6	17.7 ± 1.6	19.8 ± 0.6	17.7 ± 0.8	0.006
C20:5 n3	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	NS
C24:0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	NS
C24:1	1.4 ± 0.3	1.5 ± 0.4	1.0 ± 0.2	1.5 ± 0.3	0.039
C22:6 n3	10.0 ± 0.8	9.5 ± 1.4	11.2 ± 1.1	9.2 ± 1.3	NS

All data are shown as the mean values ± standard deviation. Percentage share of fatty acids: C13:0, C14:1, C15:1, C20:0, C20:3 n6, C20:3 n3, C22:0, C22:2, was < 0.1% and they are not included into table. For variables with skew distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as the mean and confidence interval; p value < 0.05 for those fatty acids with significant differences among groups in Kruskal-Wallis test; nd – not detected; NS – not significant differences among groups in Kruskal-Wallis test (p value > 0.05)

tent of fat in O2 was the lowest but we observed significant variations among individuals. No connection was found between liver mass and its fat content in examined groups.

Concentration of TBARS, in respect both to the mass of in liver tissue and to in its fat content, did not differed among examined groups. Moreover, in all groups great differences among individuals were observed, which suggests that the influence of applied dietary supplementation on the oxidative stress is marginal.

FAME profiles of livers were measured using gas chromatography (GC). In our experiment we analyzed 31 fatty acids. C18:0, C20:4 n6 (AA), C16:0, C18:2 n6 *cis* (LA), and C22:6 n3 (DHA) were found to be the main fatty acids in livers of all investigated groups (Table 4). There were significant differences in concentration of some fatty acids among examined groups. Those differences were caused mainly by two-step supplementation of diet with conjugated linoleic acids. In O1 we detected the highest concentration of C18:0, C20:1, AA and DHA acid and the lowest concentration of C16:0, C16:1, C18:1 n9 *cis* (OL), C18:2 n6 *trans*, LA, C18:3 n3 (ALA), C20:2 and C24:1 acids. The two-step supplementation of diet with vegetable oil increased the mean concentration of C21:0 and decreased the concentration of C20:1 in livers of K1 group. Unlimited consumption of standard fodder caused visible similarity in content of most fatty acids in livers of K2 and O2.

FAME profiles of hepatic microsomes were also investigated using GC and 29 fatty acids were identified and determined in them. The most common fatty acids in microsomes obtained from livers from animals of all experimental groups were: C18:0, C20:4 n6 (AA), C16:0, C18:2 n6 *cis* (LA) and C22:6 n3 (DHA). The concentration of many of them differed significantly among experimental groups (Table 2). Concentration of some of the saturated fatty acids (C14:0, C15:0, C16:0 and C24:0) was decreased, whereas concentration of others (C18:0) was increased by the two-step supplementation of diet with CLA. In microsomes of O1 group we also detected the lowest concentration of C16:1, OL, LA, ALA and C24:1 and the highest concentration of C20:1, C20:3 n3 and DHA. The two-step supplementation of diet with vegetable oil had also great influence on fatty acids concentration in hepatic microsomes and it increased concentration of some fatty acids (C14:0, OL, C21:0, C20:2, EPA, C24:0) and decreased the AA content.

In our experiment, we used Bio-C.L.A. as a source of CLA. As previously described, it consists of several fatty acids, mostly *trans*-10, *cis*-12 CLA (33%) and *cis*-9, *trans*-11 CLA (31%) (18). These two main CLA isomers were detected only in all livers of O1 group. In livers acquired from other investigated groups only rumenic acid (RA) was present, but in much lower amount. In O1 group, with two-step CLA supplementation, the percentage share of RA in total fatty acids amount in liver was much higher than in other groups (Table 4) and its mean content was $0.2 \pm 0.1\%$ of total fatty acids. The other CLA isomer: *trans*-10, *cis*-12 CLA constituted only $0.1 \pm 0.0\%$ of total fatty acids in livers. In microsomal fraction of hepatic tissue both CLA isomers were identified only in O1 group (Table 5). Comparison of their distribution in hepatic microsomes revealed the great similarity to their distribution in whole tissue, because *cis*-9, *trans*-11 CLA concentration tended to be much higher than *trans*-10, *cis*-12 CLA ($0.2 \pm 0.0\%$ *versus* $0.1 \pm 0.0\%$).

Our experiment allowed to test the statistical differences in dietary supplementation and the differences in supplements administration to mothers and children (K1, O1) or only to mothers (K2, O2) on parameters describing the desaturation effectiveness. Results of permutation test for individual effects and their interactions are shown in Figure 1.

Results of the analysis of the main factors and interactions are shown in Figure 2.

In applied model systems the differences in arachidonic acid concentrations between incubated and non-incubated samples indicated the enzymes' activity. The highest enzymes activity was found in K1 group, which proves that the addition of vegetable oil increases the levels of AA and increases the activity of D6D. If supplementation is carried out only for mothers (K2), this effect is less pronounced. In case of CLA supplementation, the observed effect was opposite (Table 6).

Two-step supplementation of diet with vegetable oil caused the increase in AA concentration, whereas the effect of two-step supplementation with CLA was opposite. If the supplementation was limited only to mothers, CLA increased AA concentration, whereas oil decreased it.

DISCUSSION AND CONCLUSION

The liver is a key organ in lipids metabolism. It integrates the pathways of fatty acids, triacylglycerols and phospholipids synthesis and degradation. Many processes of lipid metabolism, e.g., fatty acids esterification or desaturation, are related to microsomal fraction of cells where necessary enzymes are located (24) and therefore liver is an appropriate

Table 5. Fatty acids profile in hepatic microsomes of experimental groups.

Fatty acid	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
	[% of FA]				
C12:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	NS
C14:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.049
C15:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.010
C16:0	18.5 ± 1.4	18.2 ± 1.4	16.3 ± 1.1	18.4 ± 1.2	0.013
C16:1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.000
C17:0	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	NS
C17:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:0	26.2 ± 1.4	25.9 ± 1.3	29.0 ± 1.1	24.6 ± 1.2	0.000
C18:1 n9 *cis*	5.4 ± 0.7	4.8 ± 1.0	4.1 ± 0.3	4.7 ± 0.8	0.001
C18:2 n6 *trans*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C18:2 n6 *cis*	12.6 ± 1.4	12.6 ± 1.4	10.8 ± 1.2	13.4 ± 1.3	0.005
C18:3 n6	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	NS
C18:3 n3	0.5 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.1	0.002
C20:0	0.1 ± 0.0	0.0 (0.0-0.1)*	0.0 ± 0.0	0.0 ± 0.0	0.005
cis-9, *trans*-11 CLA	0.0 (0.0-0.0)*	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.005
trans-10, *cis*-12 CLA	nd	nd	0.1 ± 0.0	nd	NS
C20:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C21:0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.043
C20:2	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	NS
C20:4 n6	19.2 ± 2.3	20.2 ± 0.8	21.4 ± 0.9	19.8 ± 0.7	0.013
C20:3 n3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.012
C20:5 n3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	NS
C22:2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C24:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.005
C24:1	1.4 ± 0.4	1.3 ± 0.3	1.0 ± 0.2	1.4 ± 0.3	0.034
C22:6 n3	9.3 ± 1.0	9.6 ± 1.2	10.4 ± 0.9	8.99 ± 1.05	NS

All data are shown as the mean values ± standard deviation. Percentage share of fatty acids: C10:0, C14:1, C20:3 n6 was < 0.1% and they are not included in the Table. For variables with skew distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as the mean and confidence interval; p value < 0.05 for those fatty acids with significant differences among groups in Kruskal-Wallis test; nd - not detected; NS - not significant differences among groups in Kruskal-Wallis test (p value > 0.05).

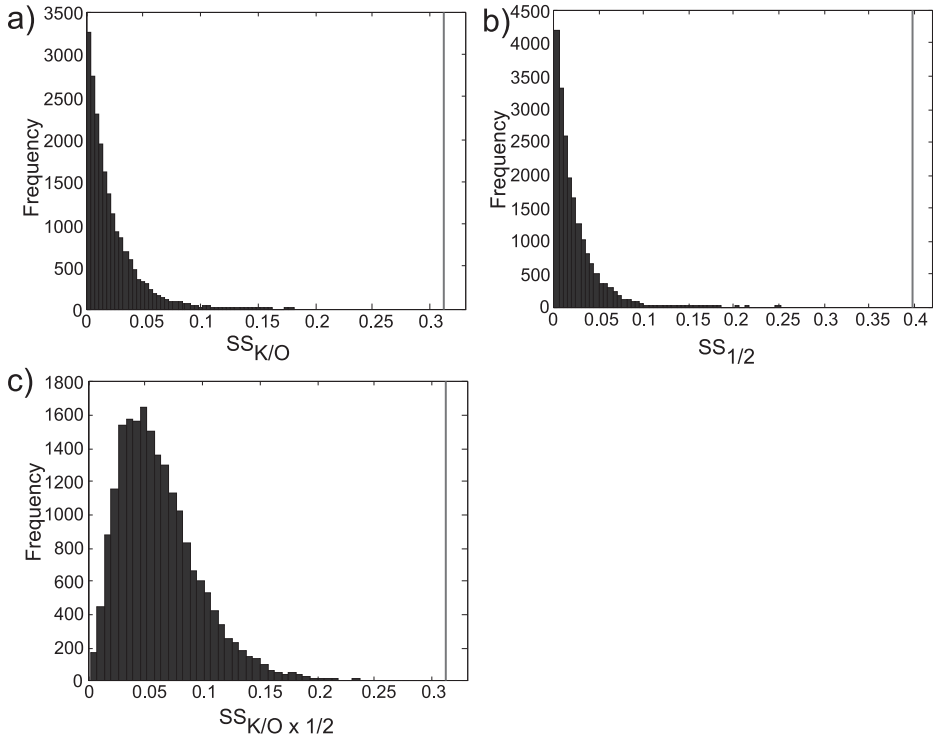

Figure 1. Results of permutation test for the various effects

organ to investigate the profile of CLA and other fatty acids (25).

Conjugated linoleic acids are a group of isomers of linoleic acid with wide range of biological activity. Their beneficial properties in many pathological conditions e.g., obesity, arteriosclerosis, cardiovascular diseases, osteoporosis, diabetes, insulin resistance, inflammation and different types of cancer are the subject of numerous scientific studies. Their ability to lower the breast cancer risk draws especially much attention. Many potential mechanisms of anticancer activity of CLA are investigated, e.g., antiproliferative, proapoptotic, antiangiogenic, antiestrogenic and competition with linoleic acid in metabolic pathway (11). Activity of Δ6- and Δ5-desaturases is the main factor which controls the conversion of LA to AA, however the effectiveness of this reactions depends not only on the activity of crucial enzymes but also on the absolute content of fatty acids, as the substrates. Since many dietary factors can regulate this pathway, we decided to check whether CLA can influence these enzymes, directly and indirectly. It seems to be especially important in cancerous process. As rats show gender differences, with females responding better than males (26), in our study we used female Sprague-Dawley rats as model organisms. We previously showed that supplementation of diet with 1.0% CLA not only decreases the risk of chemically induced breast cancer in female Sprague-Dawley rats but also influences the fatty acids profile in serum and in hepatic microsomes (19). In this study, we decided to use one or two step supplementation of diet to verify if the overall dose of CLA and the time of supplementation influenced the effectiveness of chemopreventive action.

Addition of CLA to the diet of pregnant and breastfeeding rats increases their concentration in maternal milk and as a consequence – their intake by the offspring (27, 28). The same correlation was shown for women who consumed dietary products enriched with CLA. Their milk contained much more CLA (29). We also previously proved that numerous dietary and environmental factors can elevate CLA content in maternal milk of breastfeeding women (30, 31). In our experiment, the higher supply of conjugated linoleic acids in diet of pregnant and breastfeeding females revealed the lower susceptibility to chemically induced mammary tumors in their offspring (Table 2). Effectiveness of chemo-

preventive action was comparable in both CLA groups (O1 and O2), which suggests that optimal dosage of conjugated diens of linoleic acid in early stage of life influences health in adulthood. Our results are in line with those of Ip et al. (32), who supplemented the diet of young rats for 5 weeks before the carcinogenic agent administration and demonstrated the inhibition of mammary carcinogenesis. They claimed that CLA given during the maturation of mammary glands can change their structure, e.g., diminish the number and differentiation of terminal end buds and can reduce the number of places of potential cancer induction. Moreover, numerous studies confirm the anticancerogenic properties of CLA in breast cancer model (9, 33, 34).

We observed significant differences in liver mass among examined groups. Feeding vegetable oil during the whole life resulted in increase in the liver weights, whereas influence of two-step CLA supplementation seemed to be similar but not so evident. Our observations are in line with those obtained by others. Javadi et al. (35) detected an increase in liver weights only after 12 weeks of feeding mice with CLA, but there were no differences after 3 weeks period. Akahosi et al. (36) did not observe any significant differences in inner organs weight, however, mass of livers seemed to be slightly bigger in groups of male Sprague-Dawley rats supplemented with single CLA isomers, or with their mixture. Ip et al. (9) did not note any differences in organs' mass either when the mixture of CLA isomers was applied. Long term feeding of male Fisher rats with 1.0% of CLA mixture did not change the liver weights (26). Turpeinen et al. (37), who applied single CLA isomer – *cis*-9, *trans*-11, claimed that rumenic acid did not influence the inner organs' mass. These findings are in line with those of Ip et al. (38), who used in their experiment mice with cancer, and observed that *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA caused the significant increase of mass of such organs as liver, heart and spleen.

As far as fatty acids profile in liver is concerned, our observations were similar to those of other authors. Javadi et al. (35) found the same fatty acids to be the most prominent in mice livers and confirmed that both CLA supplementation and the feeding period significantly changed the content of some of the fatty acids in examined tissue. Twelve weeks supplementation reduced the concentration of AA, 22:4 n6, C20:3 n6 and DHA, and elevated the content of C18:4 n3, C20:1 n9 and EPA. Moreover, the addition of CLA to diet changed the amount of C16:0, C18:3 n6, C20:2 n6 and C22:5 n3. The authors concluded that these changes could be the result of lower effectiveness of desaturation and elongation processes, caused by CLA. Also Kostogrys et al. (39), who checked whether CLA can reverse the harmful effect of high-fructose diet on fatty acids profile in liver, emphasized the beneficial impact of CLA on the concentration of some of the fatty acids.

As far as fatty acids concentration in microsomal fraction of livers are concerned, we detected the highest amounts of the same fatty acids as other researchers did (40–42). They proved that fatty acids profile in liver microsomes depended on the fatty acids profile in diet and dietary supplementation, especially with long chain polyunsaturated fatty acids, significantly influence not only the fatty acids composition of microsomes but also their function, e.g., activity of numerous enzymes.

We observed some interesting tendencies in fatty acids content, especially in n3 and n6 polyunsaturated fatty acids. The highest content of DHA and its substrate ALA was detected in O1, whereas their lowest content was in O2. For EPA, which is the intermediate in conversion of ALA to DHA (43), a similar result might be expected. However, we did not detect such correlation, but obtained results confirm our previous observation. CLA seem to

Table 6. Influence of the diet on AA concentration increase, D6D and D5D activity in hepatic microsomes.

Group	Increase of AA concentration [mg/100 mg of protein]	D6D	D5D
K1	0.43 ± 0.13	$(4.39 \pm 0.17) \times 10^{-3}$	1.73 ± 0.06
K2	0.13 ± 0.07	$(4.38 \pm 0.11) \times 10^{-3}$	1.71 ± 0.08
O1	0.16 ± 0.06	$(2.41 \pm 0.22) \times 10^{-3}$	1.68 ± 0.12
O2	0.13 ± 0.08	$(2.71 \pm 0.15) \times 10^{-3}$	1.71 ± 0.07

All data are shown as the mean values ± standard deviation.

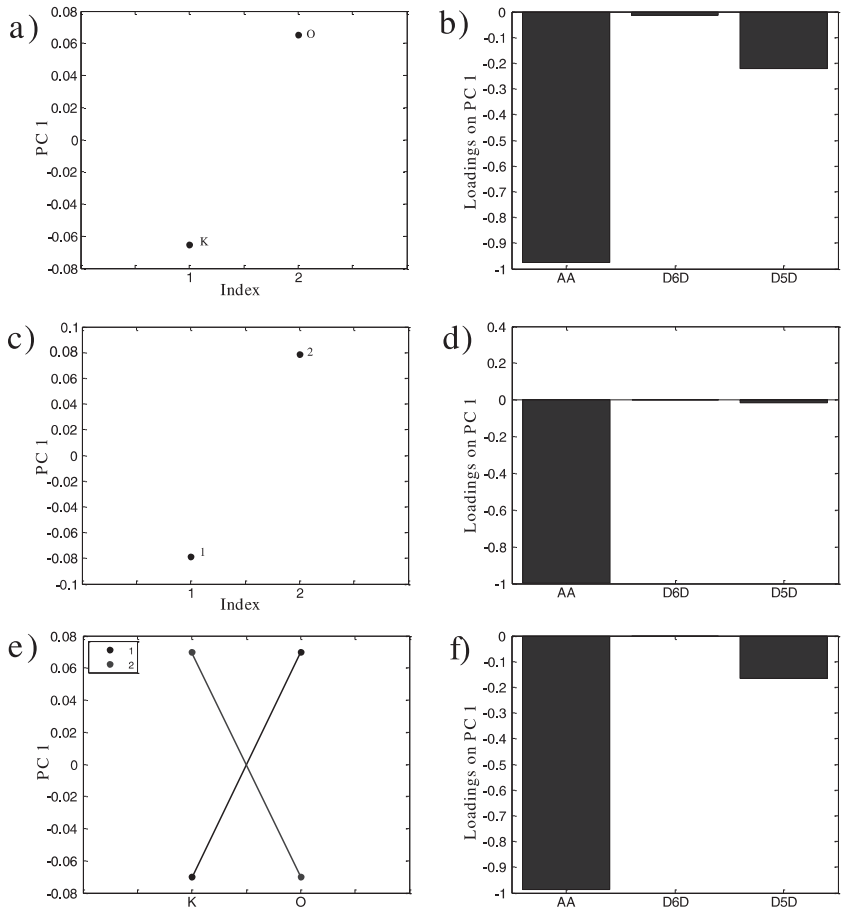

Figure 2. Results of the analysis of the principal components and their interactions

increase DHA and decrease ALA and EPA content in hepatic microsomes (19). Cao et al. (28) also observed elevated levels of DHA in liver phospholipids of suckling rats after supplementation of their mothers' diet with CLA. Also Martins et al. (25) detected similar increase in DHA content both in polar and in neutral lipids of rats' livers after CLA mixture administration, while single isomers did not exert such an effect. Eder et al. (44) observed elevated levels of DHA as well as the sum of n-3 FA in liver phospholipids as a result of CLA supplementation.

LA, which starts the n6 fatty acids family, is converted into AA, which in turn is the precursor for eicosanoids synthesis. The highest concentration of AA was detected in O1, whereas its lowest amount was in K1. These results are in line with those for LA content in microsomes, which was the highest in K1 and the lowest in O1. It confirms that supplementation of diet with CLA influences the fatty acids profile in hepatic microsomes, especially polyunsaturated n6 fatty acids. Belury et al. (45), who detected the decreased content of LA and AA in liver phospholipids in mice receiving CLA, also proved that CLA can compete with n6 fatty acids and determine their metabolism. Results of Javadi et al. (35) also confirm the ability of CLA to decrease the LA and AA content in livers of mice. Some differences in our results concerning fatty acids profiles in hepatic tissue and its microsomal fraction after CLA supplementation in relation to results obtained by others can be caused by the coexisting cancerous process. Our results indicate that not only diet but also coexisting factors such as pathological conditions or diseases e.g., cancers affect the levels of fatty acids in tissues. We previously reported (19) the significant differences in fatty acids profile in serum and hepatic microsomes of rats treated with DMBA and those not treated with DMBA. It has been observed that the profile of fatty acids in serum

of patients suffering from different types of cancer differs from distribution of fatty acids in wealthy people. Plasma phospholipids from patients suffering from bladder cancer contained much lower levels of LA and its metabolites, whereas the levels of ALA did not differ (46). There are many possible explanations for this fact but the most probable one is the changes in lipids metabolism, e.g., enhanced lipolysis or lipids peroxidation (47) or inhibited action of desaturases, mainly Δ6- desaturase, which was characteristic of cancer cells (46).

Although CLA concentration in breast tissues is generally higher than in liver (48), phospholipids of livers are richer in CLA isomers than phospholipids of mammary glands (9). Conjugated linoleic acids are preferentially incorporated into triacylglycerols rather than into phospholipids (25, 49). Liver possesses a reasonable ratio between phospholipids and neutral lipids and is an appropriate organ for studying of CLA incorporation (25). In this study, we detected both CLA isomers only in livers and in their microsomal fraction of the O1 group – constantly supplemented with CLA. In other groups only *cis*-9, *trans*-11 CLA was present in examined material, but its concentration was much lower than in O1. Although the higher supply of CLA in mothers' diet during pregnancy and breastfeeding caused only minor incorporation of CLA into hepatic tissues of children, CLA health-promoting effects were significant. *Trans*-10, *cis*-12 CLA content in hepatic microsomes and liver tissue was much lower than the content of rumenic acid despite their similar distribution in fatty acids pool of O1 group diet (Table 1). Our results are in line with those of Martins et al. (25), who detected that *cis*-9, *trans*-11 CLA was much more incorporated into both polar and neutral hepatic lipid fractions than *trans*-10, *cis*-12 CLA. However, Cao et al. (28), who used equimolar mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in two dosages (1.0 and 2.0%), found higher concentration of *trans*-10, *cis*-12 CLA isomer in liver phospholipids of both groups and in phospholipids from livers of their progeny. According to Tsuzuki (50) such diversity emerges from differences in metabolism and not in the bioavailability. *Trans*-10 *cis*-12 octadecadienoic acid activates β-oxidation and facilitates its own metabolism. Differences in CLA isomers incorporation are observed not only in liver but also in other tissues. Huot et al. (51) indicated in caveolae phospholipids of MCF-7 breast cancer cells lower amounts of *trans*-10, *cis*-12 CLA than *cis*-9, *trans*-11 CLA, despite their similar content in applied mixture. Our previous results also confirmed preferential incorporation of *cis*-9, *trans*-11 CLA into hepatic microsomes (19) and breast cancer tissues (15).

Lipid peroxidation is the result of oxidative stress and can lead to cell and tissue damage (39). Cancerous process is related to the increase in lipids peroxidation. Polyunsaturated fatty acids in phospholipids are especially prone to this reaction (52). Other sources suggest that chemopreventive properties of PUFA are the result of toxic effect of their peroxidation products on cancer cells (53). Results of numerous studies showed the increased levels of TBARS in serum of patients suffering from cancer of different organs: breast, lungs, stomach or small intestine (52, 54). However, some researchers claim that decreased lipids peroxidation stimulates the proliferation of cancer cells and promotes their malignancy (55). Many researches emphasize the antioxidative properties of CLA. Ha et al. (56) compared the antioxidative potential and concluded that CLA is as strong an antioxidant as α-tocopherole and almost as strong as butylhydroxytoluene. We did not detect any significant changes in TBARS concentration in livers among examined groups, which could confirm or deny the antioxidative properties of CLA. Sugano et al. (57) did not detect any significant differences in TBARS content in serum and in hepatic tissue after CLA addition either. Kostogrys et al. (39), who indicated the elevated MDA levels in livers after fructose administration, did not observe any influence of CLA on MDA, in comparison to control group. Ip et al. (9) also did not indicate any differences in TBARS content in livers of animals treated with CLA mixture, although they observed their decreased amount in mammary glands. Moreover, the comparison of the strongest anticancerogenic effect of 1.0% CLA and the strongest antioxidative action of 0.25% CLA separated the toxic effect on cancer cells and the potential antioxidative properties of CLA. Our results seem to confirm these findings.

The highest enzyme activities, measured by the growth of AA, D6D and D5D, was reported in K1 group. Vegetable oil, which was administered to K1, contained significantly higher amount of LA than Bio-CLA (almost three times – 27%) (19) and caused the greatest increase in enzyme activity. This indicates that supplementation of diet with oils rich in n6 fatty acids stimulates the metabolism of polyunsaturated fatty acids and formation of AA by increasing the activity of desaturases. This is turn may promote the elevated synthesis of eicosanoids, produced from AA. One of them is prostaglandin E_2 (PGE_2), which has strong immunosuppressive prop-

erties and inhibits formation of antibodies and immune cells. It also shows the pro-inflammatory and pro-tumorigenic activities (58, 59). The experimental material received from CLA supplemented animals was characterized by the significantly lower concentrations of AA and D6D in comparison to group receiving only vegetable oil. This clearly shows the inhibitory effect of conjugated linoleic acid on the activity of the enzymes, that confirms the assumption that CLA reduces the level of AA in cells directly by the influence on LA content in membranes, or indirectly, through the regulation of metabolism. Conjugated linoleic acid can be incorporated instead of LA to phospholipids and neutral fats, which are the part of cell membranes and in this way reduced the availability of the n6 substrates for the transition. However, CLA is affecting the metabolism of LA, by competing for key enzymes controlling transitions of both compounds simultaneously, which leads to decreased levels of LA metabolites, including AA and prostaglandins emerging from this fatty acid. Similar results were obtained by Bretillon et al. (60), who showed an inhibitory effect of both CLA isomers: *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on Δ6-desaturase activity. Thijssen et al. (61) in the *in vivo* studies also indicated the reduced activity of Δ6-desaturase in humans, due to the early supplementation with a mixture of CLA, while CLA had no effect on Δ5-desaturase activity. Similar results were obtained in studies with human hepatoma cell line HepG2, but only for the *trans*-10, *cis*-12 CLA (65). Also Javadi et al. (35) suggest lower desaturation and elongation activity in the liver of the CLA-fed mice. Moreover inhibited action of desaturases, mainly Δ6-desaturase, is characteristic for cancer cells (46).

It is suggested that the desaturase activity affects the inflammatory process. The increase in the concentration of AA, which correlates with the eicosanoids formulation, plays an important role in the development of inflammation (63). Reduction of the concentration of AA and its metabolites, leading to the weakening of the inflammatory response, is one of the proposed mechanism of anticancerogenic effect of CLA isomers (64–67).

Our results indicate that conjugated linoleic acids can inhibit the development of chemically induced mammary tumors. Their higher concentration in diet influence the fatty acids profile in livers and in their microsomal fraction, as well as the enzymes' activity. The decrease in D6D activity and the increase in AA concentration due to the presence of CLA in the diet of animals can confirm their anticancer properties. The higher supply of conjugated linoleic acid in mothers diet during pregnancy and breastfeeding not only causes their incorporation into tissues of children but also exerts health-promoting effect in their adult life.

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